

Enhancing single-molecule photostability by optical feedback from quantum jump detection

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We report an optical technique that yields an enhancement of single-molecule photostability by greatly suppressing photobleaching pathways which involve photoexcitation from the triplet state. This is accomplished by dynamically switching off the excitation laser when a quantum jump of the molecule to the triplet state is optically detected. The resulting improvement in photostability unambiguously confirms the importance of photoexcitation from the triplet state in photobleaching dynamics and will allow the investigation of new phenomena at the single-molecule level. © 2008 American Institute of Physics. [DOI: 10.1063/1.3013843]

During the last decade, optically based single-molecule detection has become a widely used technique¹ that has revealed many phenomena hidden in ensemble-averaged experiments, ranging from single-emitter effects in quantum optics² to insights in biophysics.³ However, photobleaching, i.e., the irreversible conversion of an optically excited organic fluorophore into a nonfluorescent entity, is a severely limiting factor in all single-molecule studies realized under ambient conditions. Many photobleaching pathways have been reported and discussed.^{4–7} Several of them begin with the molecule being in the metastable triplet state T_1 to which the molecule has a small probability to jump through intersystem crossing (ISC).⁸ Indeed, photostability can be greatly improved by engineering organic fluorophores with intrinsically low ISC rates^{9,10} or by adding triplet state quencher.⁸ Moreover, some of these pathways may involve further photoexcitation from the T_1 state.^{11,12} In that case, photobleaching dynamics should be modified by avoiding any light excitation while the molecule is in the T_1 state.

This condition can be realized through a pulsed excitation, with a pulse duration shorter than the excited-state lifetime and a repetition period longer than the triplet lifetime τ_T . For each excitation pulse, the molecule will undergo a single fluorescence cycle and, if ISC occurs, it will decay back to the ground state before the following excitation pulse. This strategy is routinely used in high-power dye lasers¹³ and has recently been applied to ensemble of molecules in the context of high-resolution fluorescence microscopy.¹⁴

We report an experiment that transposes this strategy to the single-molecule regime, leading to a strongly reduced photobleaching rate for an individual fluorophore under ambient conditions. The scheme consists of a feedback loop on the excitation laser based on the real-time detection of quantum jumps to the triplet state T_1 .

Figure 1 shows the principle of the experiment. Under light irradiation, fluorescence from an organic molecule

can be described using the three-level Perrin-Jablonski representation,⁸ with cycles occurring between the ground singlet state S_0 of the molecule and its first excited singlet state S_1 [Fig. 1(a)]. Even though the ISC from the S_1 state to the first triplet state T_1 is spin forbidden, spin-orbit interaction leads to a nonradiative decay of the molecule to this level with a small probability. As relaxation from T_1 to S_0 is also spin forbidden, the triplet-state lifetime τ_T is orders of magnitude larger than the one associated with fluorescence. The experimental setup is based on a confocal microscope working under ambient conditions. Samples are made by spin coating on a glass coverslip a solution of polymethyl methacrylate (PMMA) (1% mass in anisole) doped with dye molecules at nanomolar concentration. The result is a few tens of nanometer thick polymer coating, in which molecules are well separated and can be individually probed.¹⁵

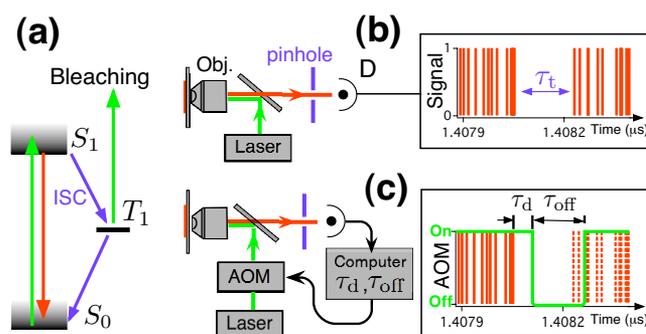


FIG. 1. (Color online) Principle of the experiment. (a) Single-molecule energy levels. (b) Photons emitted through the $S_1 \rightarrow S_0$ transition are detected with a confocal microscope. The single-molecule fluorescence time trace (b) reveals dark periods with durations on the order of the triplet-state lifetime τ_T , corresponding to quantum jumps to the triplet state by ISC. The displayed single-molecule fluorescence signal represents the output of the photon-counting detector (D) without any binning (red bars). (c) The quantum-jump detection triggers an acousto-optical modulator (AOM), used as an on-off switch for the excitation laser. If no photon is detected during a time window of duration τ_d , the laser is switched off for a duration τ_{off} longer than τ_T . The curve in green shows the AOM command for the fluorescence time trace represented in (b). The characteristic response time of the AOM is 600 ns, much shorter than all other time constants of the experiment.

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Fluorescence from a single molecule is detected by an avalanche photodiode operated in the photon-counting regime [Fig. 1(b)]. Due to the long lifetime of the triplet state, a nonradiative decay from S_1 to T_1 appears as a sudden drop in the fluorescence signal, corresponding to a quantum jump in the single-molecule emission time trace^{16,17} [Fig. 1(b)]. The fluorescence signal is fed into an electronic card that commands in real time an acousto-optical modulator (AOM) to switch off the excitation laser when a quantum jump is detected [Fig. 1(c)]. The result is a feedback loop that performs an adaptation of light excitation to the single-molecule dynamics with two adjustable time constants, τ_d and τ_{off} . As described in Fig. 1(c), τ_d is the time constant over which the decision is taken that a molecule has indeed experienced a $S_1 \rightarrow T_1$ quantum jump. This parameter acts as a temporal threshold that discriminates between fluorescence cycles and a quantum jump: if no photon is detected during τ_d , the laser is switched off for a duration τ_{off} which is set to be greater than the triplet lifetime τ_t .

The experiment is first performed with carbocyanine DiI. Single molecules are excited with a cw laser at 532 nm with an intensity of 1 kW cm^{-2} , close to the saturation of the $S_0 \rightarrow S_1$ transition. This leads to detection counting rates ranging from 30 to 200 kcounts s^{-1} , depending on the environment and the molecule dipole orientation. To limit the unavoidable bias which appears in single-molecule statistics measurements, we decide to study all fluorophores with counting rates higher than 30 kcounts s^{-1} , equivalent to a mean time interval between two consecutive detection events of $\tau_{\text{fluo}} = 33 \mu\text{s}$. In order to prevent permanent on-off switching of the excitation laser, the time constant τ_d must be greater than τ_{fluo} and is set at $70 \mu\text{s}$. Moreover, the triplet-state lifetime τ_t is approximately $200 \mu\text{s}$ for the DiI molecule. As τ_t varies depending on the local environment,¹⁷ the τ_{off} time constant of the feedback loop is set at $400 \mu\text{s}$.

For a set of single molecules, we measure the total number of detected photons before photobleaching with and without the feedback loop. We then evaluate from these data the probability $P(N)$ that a molecule has not photobleached before the record of N photocounts. The resulting probability distributions, plotted in Fig. 2(a), demonstrate a photostability enhancement when the feedback loop is applied. In addition, we measure the survival time before photobleaching,¹⁸ which is also enhanced by applying the feedback loop [Fig. 2(b)]. We note the existence of a small subset of molecules with long-lasting fluorescence, which remain unaffected by the feedback loop. This observation is consistent with the results in Ref. 17 which reports the distribution of DiI triplet-state lifetimes in a thin PMMA layer. In this reference, τ_t is smaller than $70 \mu\text{s}$ for approximately 10% of the molecules. For that subset of molecules, which are the most photostable ones, the quantum-jump-based feedback loop with $\tau_d = 70 \mu\text{s}$ will obviously be ineffective, as observed.

In order to quantify the observed gain in photostability, we then make the hypotheses that photobleaching requires further photoexcitation from the T_1 state and that there is no photobleaching when this state is empty or when the excitation laser is switched off. In this simple model which neglects all other photobleaching pathways, we also assume that after a quantum jump, the probability for the molecule to photobleach is proportional to the duration for which it remains under laser irradiation in the T_1 state. The effect of the

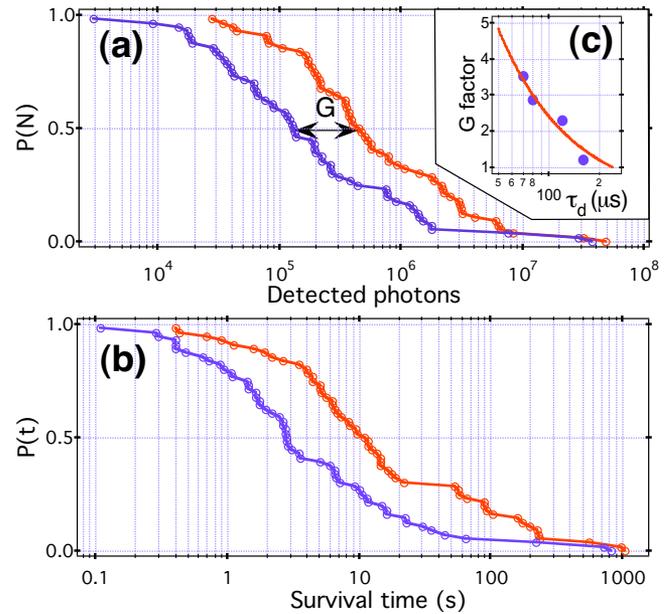


FIG. 2. (Color online) [(a) and (b)] Experimental results for DiI represented as accumulated probability distributions. $P(N)$ [$P(t)$] is the probability that a single molecule has not photobleached before the detection of N photons (before a survival time t). The statistics correspond to a set of 56 molecules without the feedback loop (blue points) and another set of 56 molecules with the feedback loop running (red points) with $\tau_d = 70 \mu\text{s}$ and $\tau_{\text{off}} = 400 \mu\text{s}$. (c) Photostability enhancement factor G as a function of the time constant τ_d of the feedback loop. For each value of τ_d , the statistics of the numbers of detected photons are measured for 50 molecules, with and without the feedback loop. The maximum value $G = 3.6$ is obtained for the probability distributions displayed in (a). The solid line represents the value of G factor given by Eq. (1) with $\langle \tau_t \rangle = 240 \mu\text{s}$.

feedback loop is a reduction in this illumination duration from τ_t to τ_d [Fig. 1(c)], leading to a photostability enhancement factor G of

$$G = \frac{\langle \tau_t \rangle}{\tau_d}, \quad (1)$$

where $\langle \tau_t \rangle$ is the average value of the triplet-state lifetime over a set of molecules in the sample.

To test our assumptions, the experiment is performed for different values of τ_d . For each set of molecules, triplet-state lifetimes are distributed with complex statistics,¹⁷ and will then correspond to different photostability gains. Therefore, for each value of τ_d we choose to estimate G as the ratio of the medians of the photocount distributions measured with and without the feedback loop. The median is known as a robust estimator of the central value of the distribution of a random variable,¹⁹ even for heavy-tailed statistics that have been found in photobleaching ensemble measurements.²⁰ The experimental results show an increase in the G factor as τ_d is decreased [Fig. 2(c)]. Equation (1) matches the data well for $\langle \tau_t \rangle = 240 \mu\text{s}$, consistent with previous measurements.^{15,17} This result provides a direct experimental verdict on the influence of triplet-state photoexcitation on photobleaching dynamics.

It is well known that photobleaching also strongly depends on the ambient atmosphere, especially the presence of oxygen.^{4,21} However, the effect of oxygen changes depending on the molecule. Whereas oxygen acts as a quencher of the triplet state for DiI, leading to an increase in the survival time before photobleaching, it enhances photodestruction in

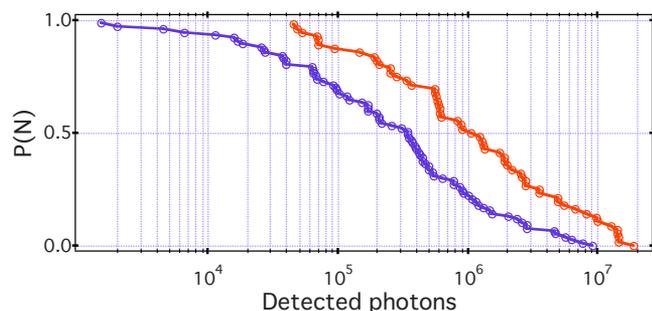


FIG. 3. (Color online) Photostability enhancement for terrylene, corresponding to $G=3.1$. Statistics are measured with a set of 72 molecules without the feedback loop (blue points) and another set of 57 molecules running the feedback loop (red points), with $\tau_d=70 \mu\text{s}$ and $\tau_{\text{off}}=400 \mu\text{s}$.

the case of aromatic hydrocarbon dyes such as terrylene.^{22,23}

To investigate a potential generality of the quantum-jump-based feedback method, the experiment is reproduced with terrylene molecules. An enhancement of both the total number of emitted photons and the single-molecule survival time is again achieved using the feedback loop (Fig. 3). A photostability enhancement $G=3.1$ is reached, consistent with the ratio $\langle\tau_t\rangle/\tau_d$.

We have shown that a simple adaptative light excitation scheme, relying on the real-time detection of single-molecule quantum jumps, leads to an enhancement of the photostability of different types of organic fluorophores that all suffer from photobleaching. This optical strategy can be operated at room temperature and under ambient atmosphere. The observed gain in photostability highlights the importance of photoexcitation from the triplet state in photobleaching dynamics. Further photostability improvement is expected by decreasing the threshold parameter τ_d in the feedback loop. This requires an increase in the detection collection efficiency or a decrease in the fluorescence lifetime, as can be achieved by coupling the fluorophore to a metallic surface²⁴ or to a nanostructured substrate.²⁵

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